Summer 2019

Gamma ray irradiation effects in soybean with regards to protein and amino acids contents of cysteine, lysine, methionine and threonine

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By

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A creative component submitted to the Graduate faculty

In partial fulfillment of the requirements for the degree of

Masters in Science

Major: Plant Breeding

Program of Study Committee:

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Iowa State University

Ames, IA

2019

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Abstract

Amino acids (AA) available in soybean meal (SBM) play a vital role in livestock feeding rations contributing to produce healthy, productive animals. The essential AA, cysteine, lysine, methionine and threonine are often added to feed as supplements from artificial sources to provide adequate ratios of AA to meet different animal species requirements. Breeding efforts for and modifications of AA content in soybean have had limited success, in part due to lack of genetic variability available for those traits and the inability to screen large populations of plants. The objective of this research was to increase genetic variability in AA content of cysteine, lysine, methionine or threonine on a well characterized variety identified as Corteva1, by applying gamma ray irradiation treatment as a mutation inducer. The irradiated Corteva1 and non-irradiated Corteva1 (used as a control) were grown at two locations adapted and non-adapted to advance generations and simultaneously determine if changes in AA composition had occurred. Estimated AA contents were obtained from the analysis conducted through near infrared spectroscopy (NIRS). Results indicated significant differences for most of the AA considered, within locations and within treatments. The comparison of AA content in the two generations analyzed, although statistically significantly different from the non-irradiated control, were small from a biological point of view. Nevertheless, two M2 plants were identified as possible candidates for future breeding efforts. Modifications to the used protocol are suggested to increase chances of identifying desirable plant candidates, and increase efficiency of use of mutagen inducers.

Abbreviations: AA, amino acid; NIRS, near-infrared spectroscopy; SBM, soybean meal; mRNA, messenger RNA; RNAi, RNA interference; EMS, ethyl-methyl-sulfonate; Gy, gray unit of gamma ray

irradiation; NaN_3, sodium azide; FT-NIR, Fourier transform near infrared spectroscopy; ILSI, International Life Science Institute.

Introduction

Soybeans [Glycine max (L.) Merr.] are utilized in many parts of the world primarily as a protein and oil source for human food, animal feed and industrial purposes (Panthee et al. 2006). In the United States, soybean is one of the most economically important crops grown (Wilson, 2008) and in 2017 it was planted on the same amount of land as maize (*Zea mays*); approximately 34% of the acreage (www.SoyStats.com, Oct. 29, 2018). From the years 1988 to 2017 the average yield of soybean in the United States increased from 1.82 t/ha to 3.3 t/ha (www.SoyStats, Oct 29, 2018) due to combined efforts in management and genetic improvement (Rinker et al., 2014).

During processing, soybeans are de-hulled increasing the relative protein content of the soybean meal (SBM) and removing the high fiber, poorly digestible seed coat (Riaz, 2006). The hull is primarily comprised of fiber. Therefore, removing this unwanted constituent allows for easier digestibility by animals. The protein content expressed in percentage becomes a higher component of the total SBM. Oil is then extracted from the crushed grain that also leaves the meal as the major source of protein.

Protein quality is directly proportionate to the amino acid (AA) composition of the chemical fraction (Panthee, 2006). Four nucleotide bases of messenger RNA (mRNA), adenine (A), cytosine (C), guanine (G) and uracil (U) are used during translation to produce codons (Pierce, 2012). In the process of translation, the nucleotides are read in groups of three bases to produce the codons of AA. There are 64 possible combinations of the four nucleotide bases when grouped into three bases at a time. Three of these codons cause translation to cease and are identified as stop codons (Ferré-D'Amaré, 2011). The other 61 codons are then responsible for the production of the 20 AA commonly found in the protein fraction of the soybean seed (National Research Council, 1989).

There are 21 AA that can form proteins in an animal body; i.e. alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, selenocysteine, serine, threonine, tryptophan, tyrosine and valine (www.nutrientsreview.com/proteins/amino-acids, July 1, 2019). Two AA, cysteine and methionine each contain a sulfur molecule and are referred to as sulfur-bearing AA (Bronsnan, 2006). For dietary purposes, the essential AA cannot be produced metabolically in the monogastric animals and they need to be consumed through food or feed. There are also conditionally essential AA which are synthesized in the body but not always in adequate amounts. There is therefore, a need to use external supplements to feed for a complete and balanced animal nutrition.

There are nine essential AA for monogastric animals, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Qi, 2010). Cysteine, lysine, methionine and threonine are found in soybean meal (SBM) at rates that still require enhancement in feed for use in livestock nutrition (Fontaine, 2003). The additional amounts of AA vary based on animal species, animal growth stage and the animals purpose of reproduction or consumption (Wang, 1989). These factors then create a priority in demand indicating which AA are the most limiting in feeding rations that will require supplementation by external sources for proper animal growth. For example, the order of limiting AA in feeding chickens is 1) methionine, 2) cysteine 3) threonine and 4) lysine (Fernandez, 1994). For swine feed, during weight gaining stages of 20-45 kg the priority changes to 1) lysine 2) threonine 3) tryptophan and 4) methionine (Roux, et al., 2011). Sauer and Ozimek (1986) studied the importance of AA digestibility in swine and reported that it is not the total amount of protein in the feed but the quality or AA content that has the largest impact on animal health. The poultry industry faces the problem of balancing lower crude protein with supplements of threonine, methionine and lysine to improve nitrogen usage and decrease excess ammonia excretion (Kidd, 1996; Toride, 2018). Currently,

the feed industry supplements the feed with external sources of AA at an added expense which is ultimately passed on to the consumer.

Lysine production for feed supplementation began in Japan in the 1960's through fermenting microbial strains grown in a medium of glucose or sugar and were extracted with an ion exchange resin treatment (Toride, 2018). Through this practice and other biological advancements, yield of artificial lysine has improved over time (Razak, 2014). Lysine plays a vital role in body protein synthesis and muscular weight gain in animals (Pacheco, 2018). As an example, Bayer Crop Science has genetically engineered a high-lysine hybrid in maize utilizing ribonucleic acid interference (RNAi) technology (Houmard, 2007). The procedure suppresses lysine catabolism specifically in the endosperm tissue of the corn kernels during the metabolic phase and ultimately increases lysine content in the corn endosperm.

Cysteine and methionine, the sulfur AA, tend to be grouped together on feed enhancement supplements (Pacheco, 2018). The reason for the grouping is because both AA are related in terms of production within the mammalian body. Methionine is the precursor to cysteine production by means of the transsulfuration pathway, which transfers a sulfur atom from methionine to serine to form cysteine. Therefore, methionine and cysteine contents must be balanced when developing animal feed. Ratios of these AA to each other, and to total crude protein are both critical to optimizing growth and minimizing feeding costs (Medic, 2014). Sulfur AA's are vital to the support of the immune system by providing molecules needed for adequate epithelial growth and lymphocyte health (Wang, 2017).

Threonine is crucial to the poultry industry, particularly to laying hens and egg production (Faria, 2002). The optimal dietary threonine content for laying hens is in the range between 0.40% to 0.58% to increase egg production and egg mass to optimal levels. An imbalance of threonine in diets of young animals reduces growth of the small intestine, liver and skeletal muscle (Wang, 2010). The imbalance

can down-regulate intestinal expression of the mucin gene which in turn reduces production of mucous in the gut, minimizing nutrient uptake and leaving the large and small intestine exposed to pathogens from the decrease in physical barrier (Wang, 2010).

Due to the lack of genetic diversity in the cultivated soybean (Hyten, 2006), the AA content of SBM is relatively stable (Thakur, 2007). In soybean, mutagenesis has proven to create useful variation in genepools and has historically proven beneficial to increase market-ability (Mudibu, 2012; Takahashi, 2003; Roychowdhury, 2013). Stolzfus et al. (2000a; and 2000b), discussed mutant alleles effects on palmitic acid content in soybean. The authors derived soybean mutants through mutagen treatment with ethyl-methyl-sulfonate (EMS) and observed elevated palmitate content in soybean oil, further concluding that this treatment was a viable option for inducing changes in fatty acid composition.

Two main types of mutagenesis used are chemical and radiation treatments (Kodym, 2003). Of these two types of mutagenesis treatments, ethyl methane sulfonate (EMS) and sodium azide (NaN_3) (Granier, 2015) are commonly used chemical mutation inducers (Kodym, 2003). X-rays, gamma rays and neutrons (fast, slow and thermal) are examples of radiation mutation inducers (Kodym, 2003). While there are pros and cons to each method, they all share commonalities such as dose, moisture content of treated material, internal oxygen level of the treated materials, whether they be whole plants, pollen grains or seeds which are variables that deserve important consideration (Oladosu, 2015).

Gamma ray irradiation was selected for the research reported here on the basis of the relative ease of seed handling post-treatment and the observation that simple chromosomal insertions or deletions are the most common effects obtained at a cellular level, rather than large genomic rearrangements (Morita, 2009). One of the most important concerns in using mutation breeding to modify seed composition has been the difficulty to screen large populations in a timely, cost effective manner. At Corteva Agriscience™ the ease of seed handling was considered one of the most important

factors for this research, since Corteva has streamlined the management processes for field research to expedite product development.

Laboratory analysis of AA is currently priced at $128.00 a sample and (https://aescl.missouri.edu/AminoAcids.html, May 25, 2019) (The University of Missouri, Columbia, MO) requires a minimum of 20g of seed per sample. For the research presented here, the average seed weight on a per plant basis was 19g per plant when grown in the adapted location (IA) and 13g per plant when grown in the non-adapted location (PR). These seed amounts would not provide enough seed for laboratory analysis and potential future propagation. Also, considering the large population sizes that would be necessary for analysis, the cost of screening would have been extensive using conventional laboratory methods for this research. These considerations guided the decision to use near-infrared spectroscopy (NIRS) for the laboratory analysis. NIRS is a commonly utilized, rapid, non-destructive method to evaluate whole soybean seeds to measure moisture, protein, oil and fatty acid composition of oil (Pazdernik et al., 1997).

The objective of this study was to determine if gamma ray irradiated soybeans selected for higher protein content would vary with regards to the content of the AAs cysteine, lysine, methionine or threonine. The laboratory method used to measure the protein and AA content was NIRS. Individual plants and advanced generations were evaluated in adapted (IA) and non-adapted (PR) growing locations.

Materials and methods

Plant materials

A proprietary soybean cultivar owned by Corteva Agriscience™, coded as Corteva1, was used in this research project. Corteva1 is a soybean variety of maturity group III with an indeterminate growth habit, best adapted to northern Missouri through central Iowa (latitudes 39.1^0N to 41.6^0N). It was

commercially available from 2001 to 2007 as a non-transgenic, high yielding soybean line, selling approximately 350,000 – 22.7 kg bags of seed in the seven years it was sold.

Within the company, Corteva1 has been used as a non-transgenic comparator for numerous research studies and regulatory trials. In these trials, its seed composition in terms of protein, oil, carbohydrate content, amino acid contents and fatty acid compositions have been determined (McNaughton, et al., 2007). Since the year 2006, Corteva1 has also been used in the development of transgenic soybean lines. Therefore, a comprehensive reference genome is available in addition to the corresponding phenotypic information. These are the reasons, that justify the use of Corteva1 as the genotype to be subjected to mutagen treatment for this research.

Mutagen treatment

Approximately 60,000 seeds of the soybean cultivar Corteva1 were subjected to mutagen treatment using gamma ray irradiation. The two treatments were applied to approximately 30,000 seeds each, conducted at Penn State College of Engineering Radiation Center (Pennsylvania State University, University Park, PA) in November 2016. Two radiation treatments were applied to the seed. In one treatment, 30,000 seeds were irradiated at a dose of 100Gy (Treatment 1). The other treatment, also of 30,000 seeds, was exposed at 200Gy (Treatment 2). The treatment doses for the research were selected based on earlier reports (Marcu, 2013; Mudibu, 2012). Previous research indicated a range of 20Gy to 500Gy would be sufficient to produce mutations possessing simple genetic alterations with limited adverse agronomic side effects. Cobalt 60 was the gamma ray source using the pool irradiator system. This protocol of gamma irradiation uses Cobalt 60 encased in aluminum or stainless steel to form pellets stored in demineralized water (www.rsec.psu.edu/Co60_Gamma_Ray_Irradiation.aspx, 12-12-18). Corteva1 was irradiated by placing seed in dry, vertical irradiation tubes 7.6 cm in diameter and submerging the tube into the demineralized pool. Treatment levels were adjusted by the proximity and

number of Cobalt 60 pellets surrounding the irradiation tubes. Remnant seed of the same batch of seed that was treated remained in cold storage at the Corteva Agriscience™ research station in Johnston, Iowa for two months and was used as control seed in all experiments conducted over the duration of the research.

Advanced generations of the mutated seed

Two locations were used for generation advances of the treated seeds and control (Table 1). One location was on the southern side of the island of Puerto Rico, outside the city of Salinas (PR). The PR research station has an annual average rainfall of 89 cm to 114 cm (www.NAOO.com, May, 25, 2019) with a climate classification of a tropical savannah, on the Köppen climate classification (www.koppen-geiger.vu-wien.ac.at/, May 25, 2019). This level of precipitation during the dry growing season is not sufficient to support healthy growth of soybeans per standard Corteva Agriscience™ best standard practices. Therefore, the use of drip irrigation during the growing season was necessary. Corteva1 is best adapted to Johnston, Iowa (IA) which is one of the main research facilities for Corteva Agriscience™. The Köppen climate classification for the IA location is a hot summer continental with annual average precipitation of 66 cm to 96 cm. Although the average precipitation for the IA location is less than that of PR, most of the water accumulation in IA occurs during the growing season and no irrigation is normally required (personal communication D. Housman Corteva Agriscience™ farm manager, 2017). Average temperature and precipitation for the two research stations was obtained from locally placed weather stations from Weather Underground (www.wunderground.com)**.**

During December 2016, 30,000 (M1) mutated seeds from each mutation treatment (100Gy and 200Gy) and 3,000 seeds of the non-mutated (M0) seed used as control were planted at the PR location (Figure 1). Off-type plants were monitored for sterility, stay-green, leaf mottling, maturity, chlorosis and

stunting of the treated plants when compared to the control. These plants were not removed from the population prior to harvest.

The seed of this generation was harvested in bulk and sent to Corteva Agriscience™ in Johnston, IA for analysis. Thirty-nine kg of the M2 100Gy seed and 36 kg of the M2 200Gy seed were analyzed. Individual seeds from each bulk were selected based on elevated protein and elevated or stable oil content when compared to the control.

In IA during June 2017, the selected M2 seeds and control seed were planted in a total of 118 plots, using the standard planting conditions at Corteva Agriscience™. In this planting, throughout the growing season phenotypically off-type plants were noticed for sterility, stay-green, leaf mottling, maturity, chlorosis and stunting when compared to the control. At the IA location in the M2 growing cycle, the off-type plants were removed prior to harvest. Individual plants were harvested from each treatment and the control during October 2017 and threshed. The number of plants harvested in the M2 and M3 plant generations are presented in Table 2.

The M3 seed was planted at PR in January 2018, with the same design as the two previous cycles. During the growing season, phenotypically off-type plants were removed prior to harvest for sterility, stay-green, leaf mottling, maturity, chlorosis or stunting. Individual M3 plants and plants from the control variety were harvested in April 2018 and sent to IA for NIRS analysis of the M4 seed from M3 plants (Figure 1).

Seed composition determination

The selection criteria for the M2 plants was based on NIRS estimations of protein and oil contents of the seed M3 seed. Selected plants had the highest protein content and stable or slightly elevated oil when compared to the control. A total of 48 M2 plants from the 100Gy and 200Gy treatments were selected based on the composition of the M3 seed for advancement to PR. Ten M0

control plants were selected based on a minimum seed number and then with all other traits considered randomly. All selections were propagated in a plant-to-row format in PR.

Fourier transform near-infrared spectroscopy (FT-NIR), a specific type of NIRS was conducted using a Bruker Tango spectrometer (www.bruker.com)(Bruker, Billerica, MA) machine to estimate oil, protein, moisture and fatty acid composition of the oil for the M3 and M4 seed of each M2 and M3 plant respectively. NIRS analysis involves collecting spectral images which can be stored and applied later with altered calibration settings. In September 2018 calibrations became available for AA content. The spectra collected from the previously scanned M2 and M3 plants were used to estimate AA content of all treated and control seeds.

Statistical analyses

Data were collected and analyzed for total protein content, and the AA composition of the protein. The variables considered for this research were contents of protein and of AA cysteine, lysine, methionine, and threonine.

The Proc Mixed function of SAS (SAS Institute, Cary, NC) was used. For each of the chemical variables the sources of variation were

$$Y_{ijkr} = \mu + jkr\Sigma_i + ikr\Sigma_j + kr\Sigma_i\Sigma_j + ki\delta_r^2\Sigma_j$$

Where,

μ = population mean

Σ_i = location effect

Σ_j = treatment effect

$\Sigma_i\Sigma_j$ = interaction term of location effect by treatment effect

$\delta_r^2\Sigma_j$ = interaction term of replication effect by treatment effect

and,

i = location = 1, 2

j = treatment = 1, 2, 3 (1 = 100Gy, 2 = 200Gy, 3 = control)

k = individual plant data = 1 to n plant

$r =$ replication = 1, 2

Analysis of variance and standard error of the mean were obtained for total protein, cysteine, lysine, methionine and threonine contents for each treatment of all plants grown at IA and PR. Irradiation treatment and location were considered fixed effects and replications were considered random effects.

Regression analysis were performed to establish the association between each of the AA considered in the research and the total protein content.

Y = α + βx

Where,

Y = an AA estimation on a dry weight basis

α = intercept

β = regression coefficient

x = total protein

Results

Protein and AA contents

The protein content of the Corteva1 control variety was significantly (P = 0.01) different between the two planting locations, IA and PR (Table 3). The difference might be related to varying environmental conditions at each growing location. The protein was 1.09%, higher at the IA location compared to the PR location. A similar trend was observed for AA content, the averages for each AA were generally higher at the IA location than at the PR location. The only exception was the cysteine content, which was 0.02 percentage points lower in content observed at the IA location compared to the PR location. The difference however, might be too small to be of biological significance. For the Corteva1 control, protein and each of the AA considered, the range of the means observed were generally lower at the PR location than at the IA location plantings (Table 3).

Irradiation treatment level effects were significantly (P = 0.01) different within locations (Table 4) and were dependent on the AA trait considered. All of the AA and even the protein contents were not significantly different among individual M2 plants harvested at the IA location when the two treatments were compared except for cysteine content. At the PR location, significant differences were observed among M3 individual plants of each irradiation levels for protein and every AA component, except for lysine content. Lysine content of the protein was similar at both of the irradiation levels. The AA cysteine had the widest variation in range observed when the strongest irradiation treatment was used and when planted at the IA location. At the PR location, the range for cysteine content was similar irrespective of the irradiation level applied to induce mutations. Despite the different irradiated seed generation planted at each location, the average protein content of plants at each treatment level and location was similar.

An important aspect to consider was the possible association between total protein and AA content (Figs. 2-5). Scatter plots were developed to establish the association and regression analyses

were performed to determine the regression coefficient at each of the planting locations between AA content in reference to total protein. The observation of each scatterplot suggests different levels of association between protein content and each of the different AA (Figs. 2-5). This is also reflected on the regression coefficients calculated for each of the AA. Lysine and threonine had the highest regression coefficients related to protein content, and the corresponding regression equations had the highest R^2 values (Figs. 3 and 5). Methionine has an association to protein content but with a regression coefficient of intermediate absolute value with a lower R^2 value compared to those of the lysine and threonine (Fig. 4). The regression coefficient between cysteine and total protein along with the R^2 value suggests that no important association was detected in this research between this AA and protein content (Fig. 2). These observations also suggest that selection for protein may not necessarily lead to an increase in each individual AA.

As a means to determine if changes in AA composition might be justified to warrant further investigation, data provided by the International Life Sciences Institute (ISLI) (www.cropcomposition.org, March 25, 2019) was used for comparison. ISLI provides access to online databases with information about AA composition on many crop species and locations. The database values are used to determine if a specific experimental protocol may have induced changes in composition that could be considered important for further research. From the comparison between AA contents measured in the research and the ISLI values, there were two outlying plants significantly outside the expected ranges. The two individual plants were identified related to the AA cysteine content evaluated at the adapted location in IA and may warrant further investigation and development. Considering the previously mentioned calculations and AA range observed among individual plants in this research, the data also suggest that it might be possible to identify additional individual plants with modified AA content that could become of significant importance.

Agronomic trait observation

Several agronomic observations of importance were also noted throughout the experiment at both planting conditions. Although these observations were not quantified, they are still considered useful data for this and future research. Germination rates appeared affected by the gamma ray irradiation treatments. As mentioned, no germination tests were performed on the treated or untreated seed, however, based on yield data obtained at the PR plots, there is evidence indicating the irradiation treatments may have affected seed germination, seedling vigor and possibly seed set of mature plants. The expectation was to receive approximately 90 kg of M2 seed from each treatment, however, only 39 kg were produced by seeds subjected to the 100Gy and 36 kg from the 200Gy treatment, respectively. The control non-treated seed nevertheless yielded as expected.

Additionally, during the M1 generation growing season, abnormalities like plant sterility, stayed-green stems at maturity, mottled leaves, maturity differences, foliar chlorosis and plant stunting were also noticed. No plants were however, eliminated from the bulk harvest during the M1 growing season. Germination rates for the IA grown M2 plants were also lower than expected compared to the untreated control; 60% and 85% respectively. In the planting at PR, plants germinated at 29% while the control seed planted had a germination rate of 58%.

Conclusions and discussion

This research was conducted with the goal to increase genetic variability for AA composition in soybean, that up to date has not been identified in nature. The specific objective was to determine if the gamma ray irradiation treatments could induce mutational changes in the AA composition of the protein fraction in the soybean seed. If this expectation was to be met, the identified mutants with modified AA composition could be of further use in breeding programs to develop soybean genotypes for marketing, as specific animal feed constituents. The results indicated that although possible, the

objective will require more time and investment, due particularly to the small differences in AA content induced by irradiation treatment effects. These considerations however, along with a detailed experimental plan will contribute to feasible and probably successful accomplishments.

The experimental procedures were evaluated as an attempt to isolate mutated plants possessing distinctive AA constitution, particularly in reference to the four AA cysteine, lysine, methionine, and threonine. In an effort to expedite mutant plant development, plantings were conducted at two different sites, the adapted location at Johnston, IA, and the off-site research station Corteva Experiment Station, at Salinas, Puerto Rico. Individual plant selections were conducted at each location. The Corteva1 non-treated seed was always used as the control genotype.

The results suggested that some experimental modifications would be necessary and probably conducive to a higher success rate. The experimental protocol used suggests that in the future it would be important to conduct the process of selection for a predetermined mutagenic trait at the adapted location, rather than at two distinctly different environments. As a means to increase seed production by individual plants, it is suggested to use the PR location to increase seed from individual plants which would produce enough seed to allow for the conducting of replicated field trials in the IA environment and possibly at several adapted locations. This procedure would allow for the identification of different experimental sources of variation that would contribute to calculating genotypic and phenotypic differences without the confounding location and environmental effects. The replicated experiments would also facilitate record keeping of desirable and undesirable agronomic traits that would be necessary to use as individual selected plants are entered into future breeding efforts and the breeding pipeline for product development.

Tables

Table 1. Environmental conditions at the M2-M3 growing locations

Growing season conditions	Adapted M2 plants (IA) 2017	Non-adapted M3 plants (PR) 2018
Average Daily (^{o}C)	22	24
Average High (^{o}C)	29	28
Average Low (^{o}C)	16	21
Precipitation (cm)	37.7	19.4[t]

[t] Precipitation supplemented twice weekly via drip irrigation

Table 2. Number of plants evaluated and selected for generation advances per each irradiation treatment at each location

	Adapted M2 plants (IA) 2017	Number of M2 plants selected for advances	Non-adapted M3 plants (PR) 2018
100 Gy	1682	21	585
200 Gy	1579	27	841
Control	428	10	579

[ǂ] Each plant selected for generation advances equates to 1 plot in Salinas.

Table 3. Average component composition, expressed on a dry weight basis, and standard error of the mean for Corteva1 M0 control seed planted at the adapted and non-adapted locations

	Planting locations		
Component[ł]	**Adapted (IA) 2017**	**Non-adapted (PR) 2018**	**Statistical significance[±]**
No. plants =	428	579	
Protein	40.13 ± 0.075	38.89 ± 0.063	**
Range	35.78 – 44.06	32.92 – 44.01	
Cysteine	0.70 ± 0.002	0.72 ± 0.002	**
Range	0.63 – 0.88	0.63 – 0.84	
Lysine	2.53 ± 0.004	2.49 ± 0.004	**
Range	2.38 – 2.78	2.00 – 2.75	
Methionine	0.59 ± 0.001	0.58 ± 0.001	**
Range	0.56 – 0.65	0.55 – 0.64	
Threonine	1.51 ± 0.002	1.46 ± 0.002	**
Range	1.43 – 1.65	1.25 – 1.60	

ł Component averages were calculated per sample size of individual plants in the year in which they were grown.

± * significant at P = 0.05, ** significant at P = 0.01 and NS = not significant

Table 4. Average component composition, calculated on a dry weight basis, with standard error of the mean and the range of 100Gy and 200Gy at adapted M2 IA (2017) and non-adapted PR M3 (2018) locations.

	Adapted M2 plants (IA) 2017			Non-adapted M3 plants (PR) 2018		
Component[ł]	**100Gy**	**200Gy**	**Statistical significance[±]**	**100Gy**	**200Gy**	**Statistical significance[±]**
No. plants =	**1682**	**1579**		**585**	**841**	
Protein	40.49 ± 0.032	40.75 ± 0.029	NS	40.53 ± 0.063	40.56 ± 0.034	**
Range	29.79 – 45.69	35.66 – 47.48		34.53 – 46.41	34.29 – 47.40	
Cysteine	0.72 ± 0.009	0.71 ± 0.001	**	0.71 ± 0.002	0.74 ± 0.001	**
Range	0.61 – 1.10	0.59 – 0.96		0.61 – 0.82	0.62 – 0.87	
Lysine	2.54 ± 0.002	2.55 ± 0.002	NS	2.59 ± 0.004	2.59 ± 0.003	NS
Range	1.87 – 2.85	2.10 – 2.89		2.17 – 2.89	1.91 – 2.92	
Methionine	0.59 ± 0.000	0.60 ± 0.000	NS	0.60 ± 0.001	0.61 ± 0.001	**
Range	0.52 – 0.67	0.55 – 0.67		0.54 – 0.65	0.55 – 0.67	
Threonine	1.52 ± 0.001	1.53 ± 0.001	NS	1.50 ± 0.002	1.51 ± 0.001	**
Range	1.25 – 1.68	1.37 – 1.70		1.34 – 1.64	1.30 – 1.67	

ł Component averages were calculated per sample size of individual plants in the year in which they were grown.

± * significant at P = 0.05, ** significant at P = 0.01 and NS = not significant

Table 5. Average component composition, calculated on a dry weight basis, with standard error of the mean and the range for 100Gy and 200Gy at the adapted M2 IA (2017) location compared to the control.

	Adapted M2 IA (2017) location				
Component[ł]	**100Gy**	**Statistical significance[±]**	**200Gy**	**Statistical significance[±]**	**Control**
No. plants =	**1682**		**1579**		**428**
Protein	40.49 ± 0.032	**	40.75 ± 0.029	**	40.14 ± 0.075
Range	29.79 – 45.69		35.66 – 47.48		36.78 – 44.06
Cysteine	0.72 ± 0.009	**	0.71 ± 0.001	NS	0.70 ± 0.002
Range	0.61 – 1.10		0.59 – 0.96		0.63 – 0.88
Lysine	2.54 ± 0.002	*	2.55 ± 0.002	**	2.53 ± 0.004
Range	1.87 – 2.85		2.10 – 2.89		2.38 – 2.78
Methionine	0.59 ± 0.000	**	0.60 ± 0.000	**	0.59 ± 0.001
Range	0.52 – 0.67		0.55 – 0.67		0.56 – 0.65
Threonine	1.52 ± 0.001	**	1.53 ± 0.001	**	1.51 ± 0.002
Range	1.25 – 1.68		1.37 – 1.70		1.43 – 1.65

ł Component averages were calculated per sample size of individual plants in the year in which they were grown.

± * significant at P = 0.05, ** significant at P = 0.01 and NS = not significant

Table 6. Average component composition calculated on a dry weight basis, with standard error of the mean and the range for 100Gy and 200Gy at the non-adapted M3 PR (2018) location compared to the control.

	Non-adapted M3 PR (2018) location				
Component[ł]	**100Gy**	**Statistical significance[±]**	**200Gy**	**Statistical significance[±]**	**Control**
No. of plants =	**585**		**841**		**579**
Protein	40.53 ± 0.063	**	40.56 ± 0.034	**	38.89 ± 0.063
Range	34.53 – 46.41		34.29 – 47.4		28.64 – 38.46
Cysteine	0.71 ± 0.002	NS	0.74 ± 0.001	**	0.72 ± 0.002
Range	0.61 – 0.82		0.62 – 0.87		0.63 – 0.84
Lysine	2.59 ± 0.004	**	2.59 ± 0.003	**	2.49 ± 0.004
Range	2.17 – 2.89		1.91 – 2.92		2.00 – 2.75
Methionine	0.60 ± 0.001	**	0.61 ± 0.001	**	0.58 ± 0.001
Range	0.54 – 0.65		0.55 – 0.67		0.55 – 0.64
Threonine	1.50 ± 0.002	**	1.51 ± 0.001	**	1.46 ± 0.002
Range	1.34 – 1.64		1.30 – 1.67		1.25 – 1.60

ł Component averages were calculated per sample size of individual plants in the year in which they were grown.

± * significant at P = 0.05, ** significant at P = 0.01 and NS = not significant

Figures

Figure 1. Generation advances of M1-M4 seed

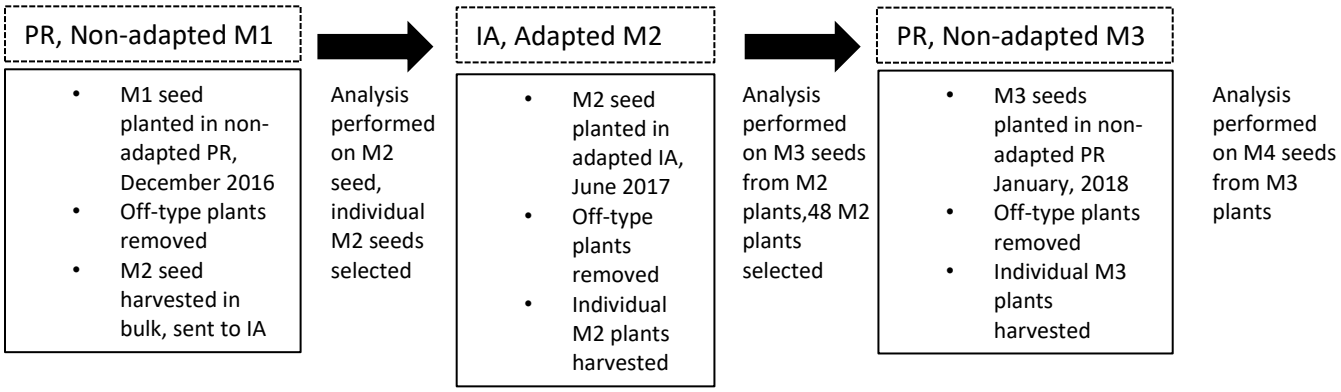


Figure 2. Scatterplot of cysteine vs. protein for IA 2017 M3 seed and for PR 2018 M4 seed per seed treatments. Regression line, regression estimates and R^2 were calculated.

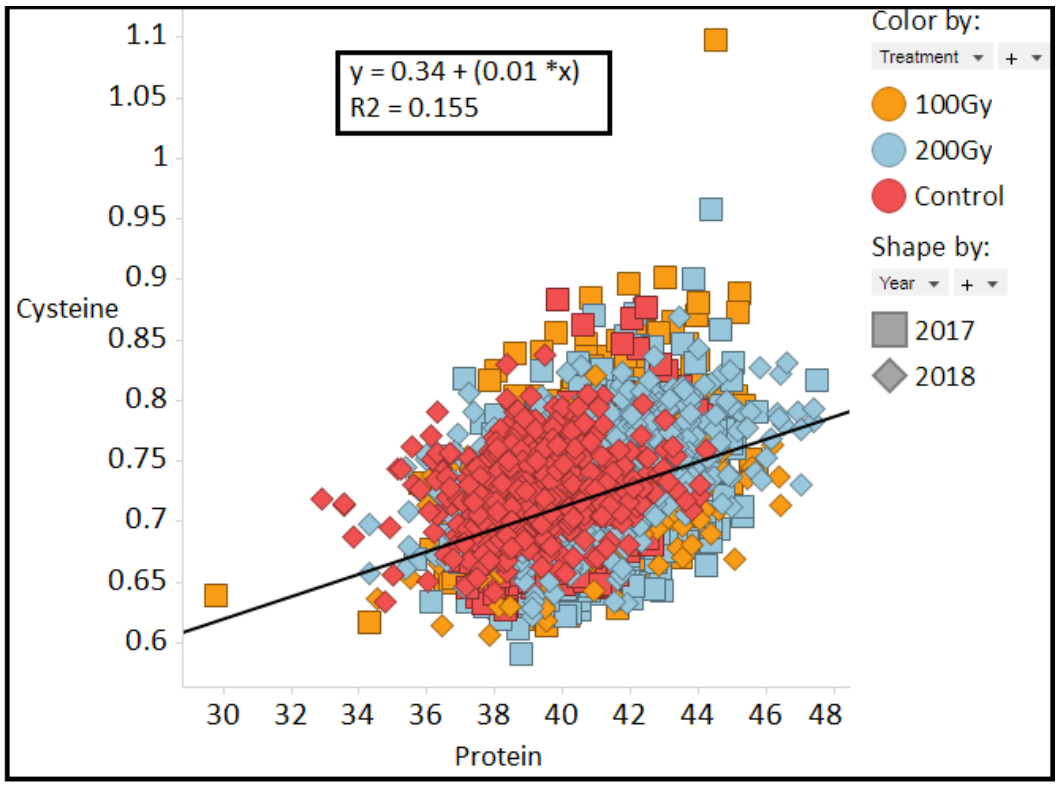


Figure 3. Scatterplot of lysine vs. protein for IA 2017 M3 seed and for PR 2018 M4 seed per seed treatments. Regression line, regression estimates and R^2 were calculated.

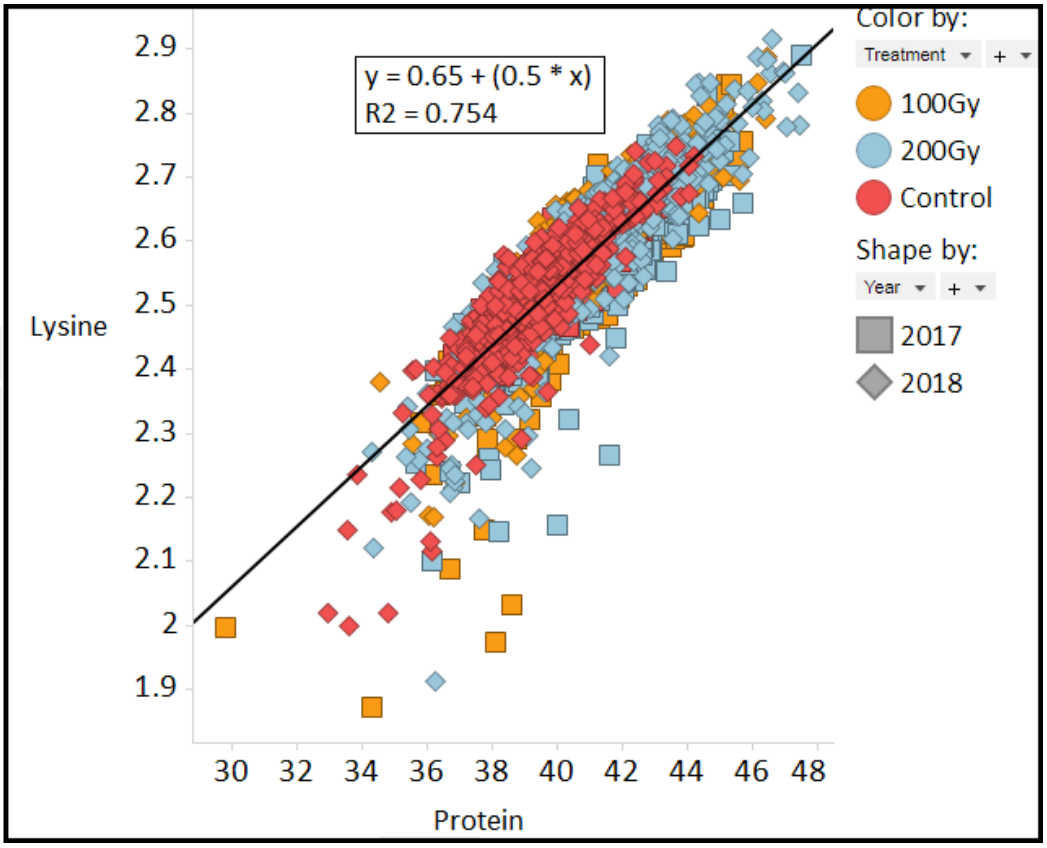


Figure 4. Scatterplot of methionine vs. protein for IA 2017 M3 seed and for PR 2018 M4 seed per seed treatments. Regression line, regression estimates and R^2 were calculated.

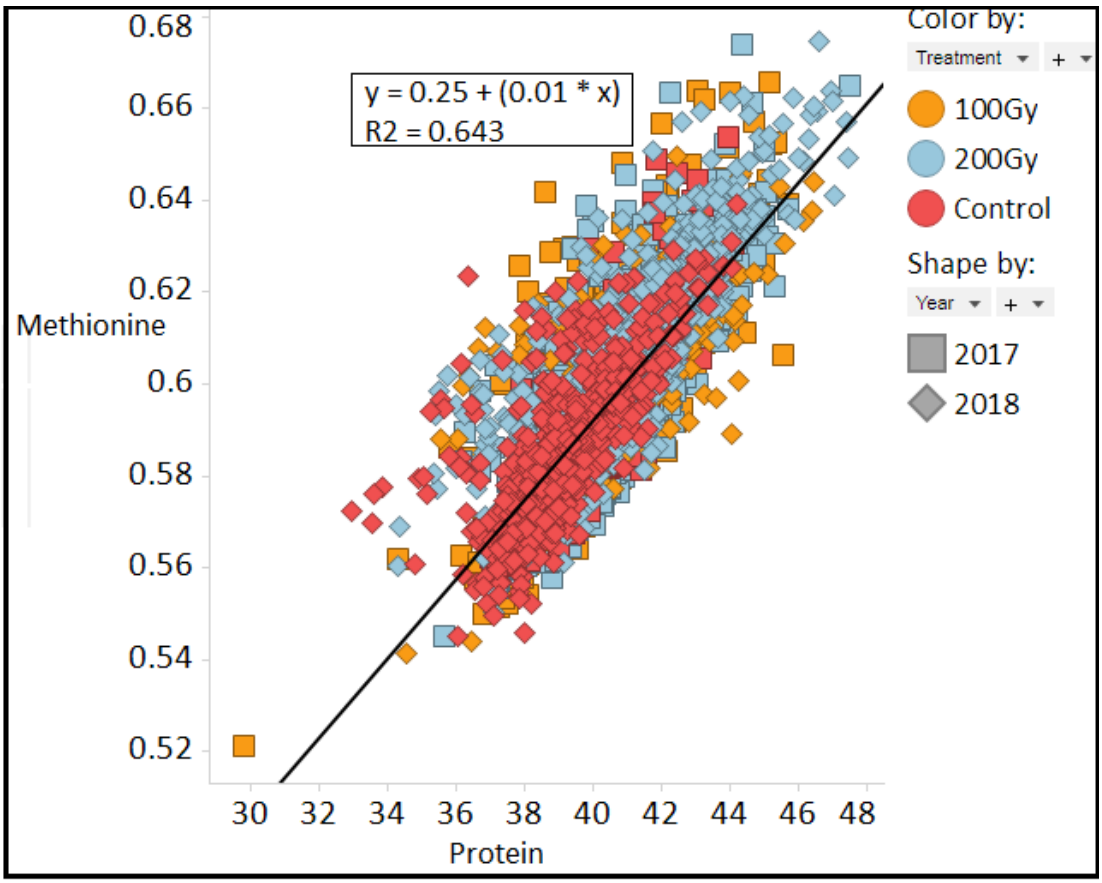
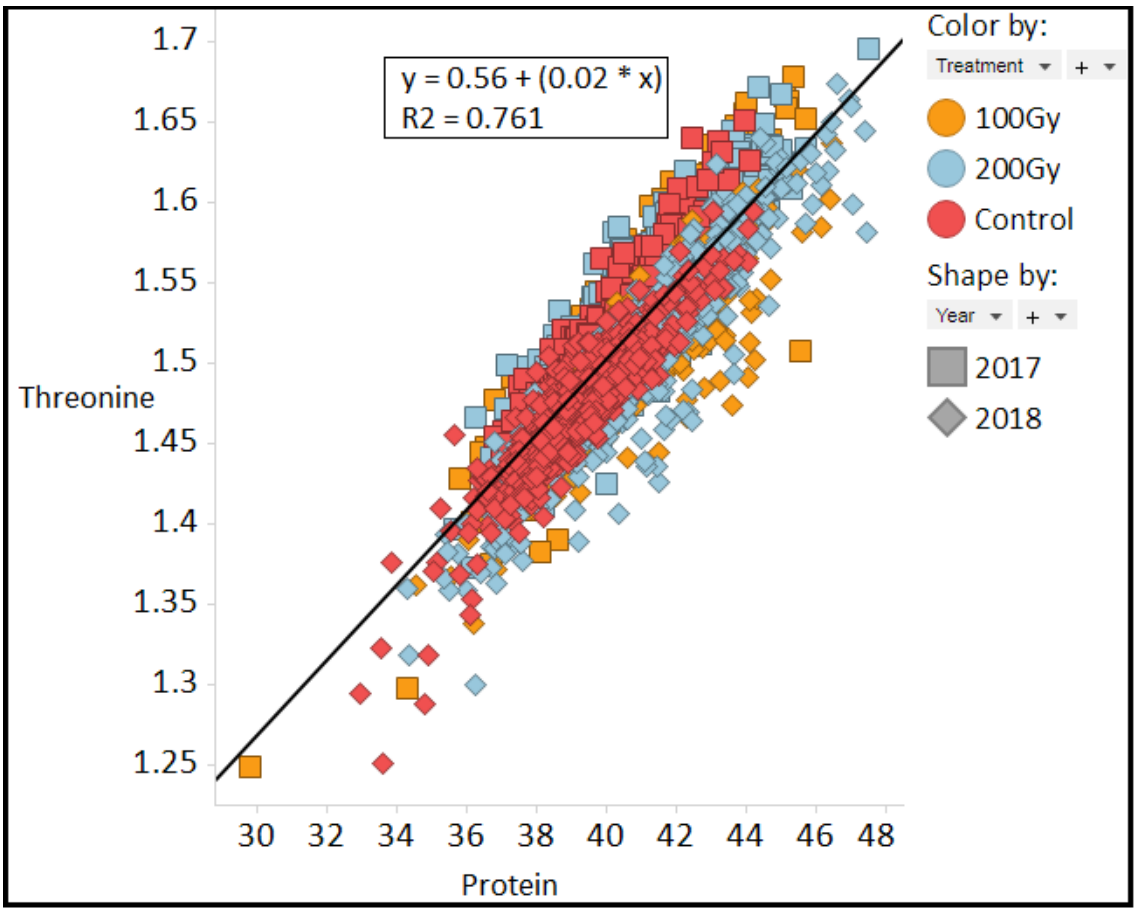


Figure 5. Scatterplot of threonine vs. protein for IA 2017 M3 seed and for PR 2018 M4 seed per seed treatments. Regression line, regression estimates and R^2 were calculated.



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Acknowledgments

There are many people I would like to acknowledge who have helped me earn this honorable degree.

The first person I would like to thank is my husband, Clay. Your patience and understanding have been more meaningful than I can put to words.

Secondly, I would like to thank Jeffery Hegstad and Mark Vogt for encouraging me to begin coursework in the distance program for this degree. Their guidance in the initial phases of my career has been critical to my current success.

Thirdly, this project would have not been possible without Bo Shen, John Everard, Russ Booth and the entire Output Traits team participation at Corteva Agriscience™. The opportunity to learn from each of you has been and will continue to be invaluable.

Lastly, I would like to sincerely thank my Major Professor, Dr. Silvia R. Cianzio for her patience with my writing skills and for guidance this last year.